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REMARKS

Election/Restrictions

The Office Communication dated October 4, 2010, indicates that the Amendment filed July 21, 2010, is non-responsive because the presented claims are drawn to the non-elected claims of Group II; and that there was no misunderstanding with respect to the election of Group I. [The January 21, 2010 Office Action, 2010, had included a requirement for a group election and species election.] Applicants apologize for the misunderstanding.

Group I is drawn to a method of inhibiting tumor angiogenesis comprising providing to cells that undergo angiogenesis or participate in angiogenesis, an effective amount or amounts of (a) one or more of thrombospondin-1 (TSP-1), an antiangiogenic derivative thereof, or a TSP-1 agonist or mimic; and in combination with (b) one or more inhibitors of the action or expression of (i) HGF/SF receptor Met, (ii) VEGF or the VEGF receptor; and (iii) both (i) and (ii), thereby inhibiting said angiogenesis.

The Office Action also indicates that a species election is required as the species identified do not relate to a single general inventive concept under PCT Rule 13.1 and 13.2. Applicants elect the species of (a) TSP-1 or an TSP-1 agonist in combination with one or more inhibitors of the action or expression of (i) HGF/SF of the HGF/SF receptor Met, (ii) VEGF of the VEGF receptor, or (iii) both (i) and (ii) (section 4), TSP-1 or anti-angiogenic derivative (section 5), an inhibitor of VEGF (section 6), and a VEGF inhibitor anti-VEGF antibody (section 7).

Applicants elect to proceed with prosecution of the claims of Group I, which includes currently pending claims 1, 5, 7-13, 16-18, 20, and 21. In view of the group election and the

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species election, claims 9, 10, 13, 16-18, 22, 26, 28-34, 37, 38, and 40-46 have been withdrawn.

As such, pending claims include 1, 5, 7, 8, 11, 12, 20, and 21.

Claim Rejection Under 35 U.S.C. § 112

The Office Action indicates that claim 8 does not comply with 35 U.S.C. § 112, second paragraph, because the claim identifies the trademark Avastin® (Office Action, p. 10).

Applicants have amended claims 8 and 29 to recite the monoclonal antibody in the products, namely, bevacizumab.

Claim Rejection Under 35 U.S.C. § 102(e)

The Office Action rejected claims 1, 5, 7, 8, 11, 12, 20, and 21 over Stein et al. U.S. Patent No. 7,351,729 (“the Stein el al. patent”). Specifically, the Office Action indicates that Stein et al. teach a method of administering one or more angiogenesis inhibitors including anti-VEGF antibody bevacizumab and TSP-1 to treat cancer (Office Action, at p. 12).

The Stein et al. patent claims an effective filing date of March 8, 2002. However, Applicants herewith submit (a) a (first) Declaration Under 37 C.F.R. § 1.131 and (b) a (second) Declaration of Livnat Under 37 C.F.R. § 1.131 indicating that the inventions of claims 1, 5, 7, 8, 11, 12, 20, and 21 were conceived prior to March 8, 2002, and Applicants were reasonably diligent in reducing the invention to practice from prior to March 8, 2002, until the filing of Applicants’ priority application on July 7, 2003. Also, attached to the (first) Declaration Under 37 C.F.R. § 1.131 are documents showing experiments from the laboratory notebook of Yu-Wen Zhang, one of the inventors of the present application. Because Applicants have antedated the Stein et al. patent, Applicants respectfully submit that the reference should not be applied against claims 1, 5, 7, 8, 11, 12, 20, and 21 of the present application and the rejection based on this

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reference should be withdrawn.

Further, even if the Stein et al. patent is applied as prior art, Applicants respectfully contend that the disclosure of this reference relied on in the Office Action (Col. 58, lines 16-41) does not anticipate claims 1, 5, 7, 8, 11, 12, 20, and 21 of the present application. In particular, the combination claimed by Applicants (TSP-1 and a VEGF inhibitor) is not specifically named in the Stein et al. reference. Instead, the Stein et al. patent indicates that a JNK inhibitor may be combined with one or more angiogenesis inhibitors from a list of sixty-three (63) agents. First, a combination of TSP-1 and a VEGF inhibitor is not identified in the Stein et al. patent because all of the agents in the list necessarily are combined with a JNK inhibitor. Second, even if the JNK inhibitor was somehow excluded from the combination, there are so many possible composition combinations that one of ordinary skill in the art cannot “at once envisage” the specific combination of TSP-1 and a VEGF inhibitor. (See, MPEP §2131.02, third subsection). Instead, the number of possible combinations is vast. The Stein et al. disclosure can include each of the 63 agents alone, combinations of 2 agents selected from the 63 agents, combinations of 3 agents selected from the 63 agents, combinations of 4 agents selected from the 63 agents, combinations of 5 agents selected from the 63 agents, combinations of 6 agents selected from the 63 agents, etc. In total, there are 63 factorial (63!) possible combinations disclosed by Stein et al.; a vast number to be sure. Accordingly, Applicants respectfully submit claims 1, 5, 7, 8, 11, 12, 20, and 21 of the present application are not anticipated by the Stein el al. patent, and the rejection based on this reference should be withdrawn.

Claim Rejection Under 35 U.S.C. § 103

The Office Action rejected Claims 1, 5, 7, 8, 11, 12, 20, and 21 under 35 U.S.C. §103(a)

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as being unpatentable over Rosen (*Cancer Control*, March/April 2002, 9:36-44) in view of Lawler US Patent 7,223,731, Streit et al. (*American J. Pathology*, 199, 155:441-452), and Burke et al. (*Critical Reviews in Oncology*, 2001, 39: 155-171).

The Office Action indicates Rosen teach administering an anti-VEGF antibody, and that TSP-1 is a known anti-angiogenic factor, but do not teach a combination of TSP-1 and a VEGF inhibitor; however the Lawler '731 patent and the Streit et al. reference disclose administering TSP-1 to treat cancer or to inhibit angiogenesis (Office Action, p. 13). The Office Action further states that Burke et al. (generally) disclose combining anti-angiogenic agents to produce a synergistic combination. As noted in the Office Action (p. 14), none of the references specifically teach combining together TSP-1 and a VEGF inhibitor; but the Office Action also state this combination would have logically followed from these agents having been individually taught for the same purpose (Office Action, pp. 14-15). However, as discussed above, even if we limit potential anti-angogenic agents to the 63 identified by Stein et al., there are an unlimited number of combinations of anti-angiogenic agents that might possibly have a synergistic effect; and the majority of these agents likely target different molecular mechanisms. It is not obvious to try this combination where the prior art gives no direction as to which of many possible choices is likely to be successful. *In re Kubin*, 561 F.3d 1351, 1359, 90 U.S.P.Q.2d 1417 (2009), The number of possible combinations is vast, and the effect of these combinations unpredictable. *Id.* Moreover, there is no reason for one of skill in the art to predict any one combination would have a synergistic effect. Here, the synergy identified by Applicants was further demonstrated by Filleur et al. [*Cancer Research*, 63, 3919-3922, July 15, 2003, Exhibit A hereto]. In particular, Filleur show the combination of anti-angiogenic agents produce synergistic effects (see, Filleur,

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p. 3922, entire Column 1).

Based upon the above, Applicants respectfully contend that it would not have been obvious for a person of ordinary skill to combine Rosen, the Lawler '731 patent, Streit et al., and Burke et al. to obtain the invention of claim 1. Because claims 5, 7, 8, 11, 12, 20, and 21 depend from claim 1 and, therefore, include additional claim elements, Applicants also respectfully contend that claims 5, 7, 8, 11, 12, 20, and 21 are non-obvious over the cited combination of references.

Applicants respectfully request that this Section 103 rejection be withdrawn and claims 1, 5, 7, 8, 11, 12, 20, and 21 allowed.

Conclusion

If the Examining Attorney has any questions regarding this election, Applicants respectfully request that the Examining Attorney contact the undersigned at the telephone number indicated below or email the undersigned at dsiegel@priceheneveld.com.

Respectfully submitted,

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Date

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SiRNA-mediated Inhibition of Vascular Endothelial Growth Factor Severely Limits Tumor Resistance to Antiangiogenic Thrombospondin-1 and Slows Tumor Vascularization and Growth¹

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Abstract

In the past few years, several laboratories have developed antiangiogenic molecules that starve tumors by targeting their vasculature and we have shown that, when produced in tumors, the antiangiogenic molecule thrombospondin-1 (TSP1) reduces the vascularization and delays tumor onset. Yet over time, tumor cells producing active TSP1 do eventually form exponentially growing tumors. These tumors are composed of cells secreting unusually high amounts of the angiogenic stimulator vascular endothelial growth factor (VEGF) that are sufficient to overcome the inhibitory TSP1. Here, we use short double-stranded RNA (siRNA) to trigger RNA interference and thereby impair the synthesis of VEGF and ask if this inability to produce VEGF prevents the development of TSP1 resistance. Systemic *in vivo* administration of crude anti-VEGF siRNA reduced the growth of unaltered fibrosarcoma tumor cells, and when the anti-VEGF siRNA was expressed from tumor cells themselves, such inhibition was synergistic with the inhibitory effects derived from TSP1 secretion by the tumor cells. Anti-VEGF siRNA delayed the emergence of TSP1-resistant tumors and strikingly reduced their subsequent growth rate.

Introduction

The observation that tumor growth is highly dependent on the ability of tumors to induce their own vascularization has led numerous laboratories to isolate or develop angiogenesis inhibitors such as TSP1⁴ (1). This antivascular strategy that targets the normal, genetically stable endothelial cells of the host rather than the genetically unstable tumor cell population was shown to be very efficient at reducing tumor growth and was not expected to trigger tumor resistance (2). However, recently we (3) and others (4) have demonstrated that changes in the tumor cells themselves, particularly sustained high-level secretion of the angiogenic stimulator VEGF, can enable tumors to bypass antiangiogenic treatments.

It has recently been shown that the introduction in a mammalian cell of double-stranded oligoribonucleotides, also called siRNA, triggers the degradation of the endogenous mRNA to which the siRNA hybridizes (5). This mechanism is highly sequence specific and allows to turn off the expression of a target protein (6, 7). Many studies demonstrated the high efficiency and versatility of RNA interference in cell cultures. Some authors developed vectors or viruses to produce

siRNA in cells (8). The *in vivo* regulation of a gene by RNA interference has been obtained either using these vectors or viruses (9) or using the so-called hyperpressure technique (10, 11), which drives siRNA mainly in the liver and would not be possible to use in humans. In this work, we demonstrate that low doses of siRNA administrated by a systemic route penetrate into tumors and control the expression of target genes to produce phenotypic effects.

The aim of the present work was to determine whether blocking the ability of tumor cells to secrete high levels of VEGF by the *in vivo* administration of siRNA could diminish or prevent the triggering of resistance to the antitumor effects of TSP1.

Materials and Methods

Cell Culture. The rat fibrosarcoma cJ4 cells (12) were grown as described previously. A bidirectional TSP1-luciferase-inducible expression vector was introduced in cJ4 cells to generate JT8 cells as described previously (3). Cells were grown in the presence of 100 ng/ml dox, a tetracycline analogue to repress TSP1 expression.

siRNA. The siRNA (sense and antisense strands) were purchased from MWG Biotech (Ebersberg, Germany). The sense strands sequences were the following: VEGF, 5'-AUGUGAAUGCAGACCAAAGAA-TT; CONT, 5'-GAUAGCAAUGACGAAUGCCUA-TT; and LUC, 5'-AACGUACGGCGGAAUACUUCGA-TT. *In vitro* transfections were performed using the TransIT-KO polymer/lipid from Mirus (Madison, WI) as recommended. For 6×10^6 cells in 10 ml of medium, 2 μ g of siRNA were used. Cells were washed 24 h after transfection.

Tumorigenicity Assays. cJ4 or JT8 cells were injected s.c. in PBS (10^6 cells/site) into the hind quarters of four to six female Swiss nu/nu mice, 4–6 weeks old (Iffa Credo, L'Arbresle, France) for each tested condition. Each experiment was repeated at least twice. When stated, dox (100 μ g/ml) was added to the drinking supply of the animals to repress TSP1 and luciferase expressions. The drinking supply was changed three times a week. Tumor volume was calculated as $v = L \times l^2 \times 0.52$, where L and l represent the larger and the smaller tumor diameter measured daily. For *in vivo* injections, each animal was injected daily with 50 (i.p., i.v., or s.c. injections) or 10 μ l (intratumoral injections) of PBS containing 3 μ g of siRNA (125 μ g/kg/day). The care of the animals was provided in the animal quarters of the Institut André Lwoff in Villejuif according to the institutional guidelines.

Immunohistochemistry, Scoring of Microvessel Density. TSP1, VEGF, and CD31 detection and scoring of blood vessels density in tumors were performed as described previously (3).

Luciferase Activity. Tumors were homogenized with a polytron homogenizer in cell culture lysis reagent (Promega). Protein concentration was measured using BSA as standard with the Bio-Rad DC protein assay. Luciferase activity was quantified in a luminometer (Analytical Luminescence Laboratories) using 1 mM luciferine as substrate.

VEGF Quantification. VEGF was quantified in cell supernatants or in tumor homogenates using an ELISA kit for mouse VEGF from R&D. Cells were transfected with VEGF- or control-siRNA. Twenty-four h later, cells were replated at the same density and conditioned media collected on day 4 after transfection. Cells were replated at equal density on day 4 and media collected on day 6 and finally replated on day 11 and media collected on day

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⁴ The abbreviations used are: TSP1, thrombospondin-1; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA; dox, doxycycline.

13. Values are expressed as percentage of the VEGF content in the CONT-siRNA-transfected cells medium collected on the same day. VEGF in tumor homogenates is expressed as pg/mg of total protein.

Results

Inhibition of VEGF Synthesis by RNA Interference *in Vitro*. siRNA matching a 21-nt sequence conserved between the human, rat, and mice VEGF A mRNA was synthesized (VEGFsiRNA). As controls, we used either a sequence presenting no significant homology with mRNA databases (CONTsiRNA) or a siRNA against luciferase mRNA (LUCsiRNA).

Transfection of VEGFsiRNA in cJ4 rat fibrosarcoma cell line (12) induced a marked reduction in VEGF synthesis and secretion (Fig. 1*a*, bottom panel, and *b*) as compared with cells transfected with the CONTsiRNA (Fig. 1*a*, left panel). The secreted VEGF level was still reduced by 50% 13 days after transfection. Untransfected, CONTsiRNA-, or VEGFsiRNA-transfected cells were engrafted s.c. to nude mice and tumor growth monitored (Fig. 1*c*). On day 12, animals were sacrificed and tumors collected. Immunodetection of VEGF in the tumors showed a marked reduction in the VEGF expression of tumors growing from VEGFsiRNA-transfected cells (Fig. 1*d*, right panel) as compared with controls. This reduction was accompanied by a 67% reduction in tumor volume. These data indicated that the reduction in VEGF synthesis obtained by the siRNA transfection *in vitro* resulted in the expected biological effects on VEGF production and tumor growth *in vivo*. The tumor growth was unaffected by the transfection of control siRNA. The logarithmic regression analysis of the tumor growth curves (Fig. 1*c*, inset) shows that the onset of tumors that express only residual VEGF levels were delayed as compared with controls but eventually grew with similar growth rates.

Delivery of siRNA to Tumors *in Vivo*. To monitor the delivery of siRNA to tumors by systemic administration, we first implanted in nude mice cJ4-derived cells constitutively expressing luciferase.

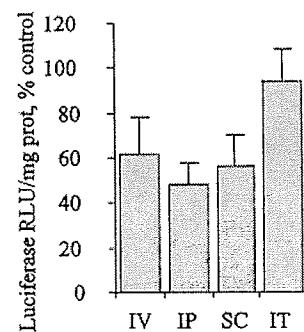


Fig. 2. *In vivo* administration of siRNA. A cJ4-derived cell line expressing luciferase was injected into nude mice. When tumors reached $\sim 200 \text{ mm}^3$, crude CONTsiRNA or LUCsiRNA was injected either i.v., i.p., s.c., or in the tumor (i.t.). Three days later, tumors were collected and homogenized to quantify the luciferase activity. Results (RLU/mg protein, mean \pm SE, $n = 3$) are expressed as percentage of CONTsiRNA-injected animals. This experiment was repeated with similar results.

When tumors reached $\sim 200 \text{ mm}^3$, mice were separated into groups of three, and each mouse received a single injection of LUCsiRNA or CONTsiRNA. siRNA was administered either via the tail vein i.v., i.p., or s.c. ($3 \mu\text{g}$ in $50 \mu\text{l}$ of saline in each case) or directly in the tumor ($3 \mu\text{g}$ in $20 \mu\text{l}$ of saline). Three days later, animals were sacrificed, tumors collected, homogenized, and luciferase activity and protein content were determined. The three systemic administration procedures induced $\sim 50\%$ inhibition of the luciferase expression (Fig. 2), whereas the direct intratumoral injection was ineffective.

VEGF Inhibition by RNA Interference *in Vivo*. Recently, using the same parental fibrosarcoma line, we established JT8 cells where the TSP1 sequence is expressed from a bidirectional vector in which a common tet operator controls TSP1 and luciferase. So, *in vitro* as *in vivo*, in the absence of dox (a tetracycline analogue), TSP1 and luciferase are expressed, whereas when dox is added either to the medium of the cultured cells or in the drinking supply of the animals

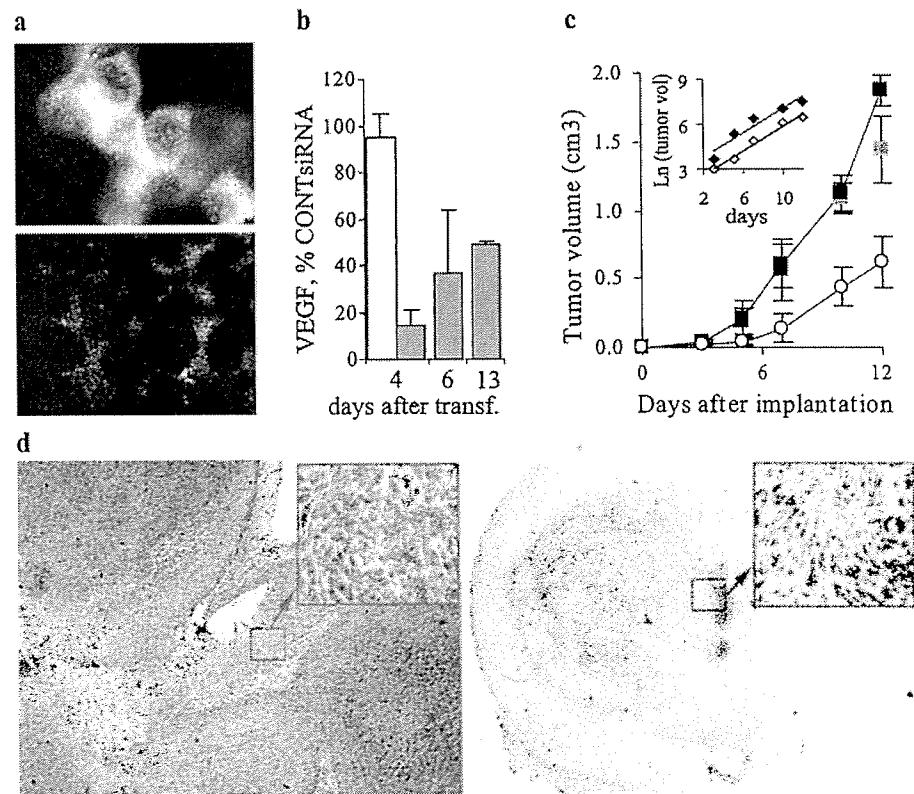
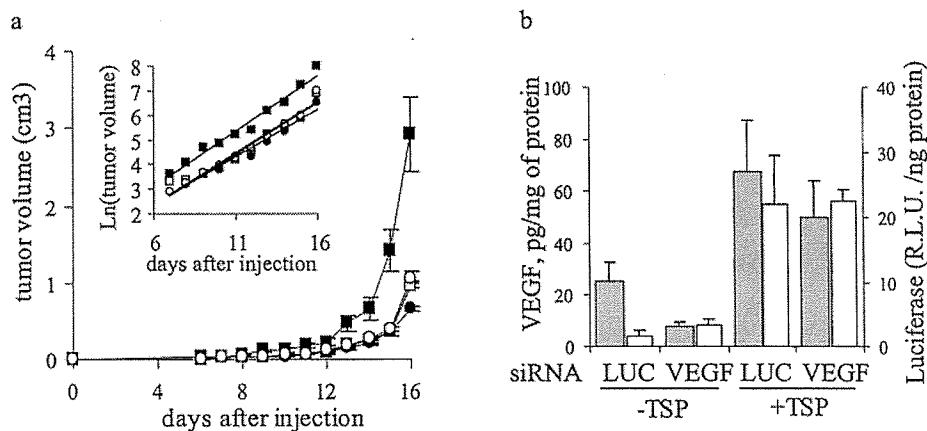


Fig. 1. Inhibition of VEGF synthesis by transfection of siRNA. *a*, control- (top panel) or VEGF-siRNA (bottom panel) was transfected into cJ4 rat fibrosarcoma cells. Two days after transfection, VEGF was detected by indirect immunofluorescence. *b*, secreted VEGF was quantified in the supernatants of cells from *a* at the indicated days after transfection. Values, measured in triplicates (\pm SE), are expressed as percentage of CONT-siRNA-transfected cells at each time point. \square , untransfected cells. The experience was repeated twice with similar results. *c*, one million of untransfected cJ4 cells (\square), CONTsiRNA- (\blacksquare) or VEGFsiRNA-transfected cells (\circ) were injected to nude mice. The tumor volume was monitored for 12 days (mean volume \pm SE, $n = 4$). *Inset*: corresponding logarithmic regression trend lines. *d*, at the end of the experiment described in *c*, tumors were fixed and immunolabelled for VEGF. A high VEGF expression was detected in tumors derived from CONTsiRNA-transfected cells (left) and only detected in few cells in tumors derived from VEGFsiRNA-transfected cells (right).

Fig. 3. *In vivo* effect of VEGFsiRNA. *a*, the tet bidirectionally inducible TSP1-luciferase JT8 cell line was injected to nude mice. Mice were separated in four groups. Groups A (VEGF+/TSP1-, ■) and B (VEGF-/TSP1-, □) received dox to block TSP1 and luciferase expression. Groups C (VEGF+/TSP1+, ●) and D (VEGF-/TSP1+, ○) received no dox. Groups A and C received daily i.p. injections of LUCsiRNA, whereas groups B and D received VEGFsiRNA. Tumor growth was monitored daily (mean volume \pm SE, $n = 4$). *Inset*: corresponding logarithmic regression trend lines. *b*, VEGF and luciferase quantification in tumor homogenates. At the end of the experiment in *a*, tumors were collected and homogenized to measure VEGF (pg/mg protein) content and luciferase activity (arbitrary luminometric units/ng protein).



if the cells are growing *in vivo*, both genes are repressed. We assayed the effect of VEGF-siRNA delivered daily (125 μ g/kg/day) by i.p. administration on the growth of JT8 cells grafted s.c. to nude mice receiving dox that insured TSP1 and luciferase were repressed. By day 16, the volume of tumors growing in such treated animals (group B) was reduced by 66% as compared with the controls (group A), which had been treated with LUCsiRNA (Fig. 3*a*). A 70% reduction in VEGF expression was measured by ELISA in homogenates of tumors from the VEGFsiRNA-treated group (Fig. 3*b*). To control that this reduction was not resulting from a nonspecific siRNA effect, the luciferase activity was measured in the same tumor homogenates. As expected in the presence of dox, this activity was low but measurable with a luminometric assay. We measured a 52% reduction in luciferase activity in LUCsiRNA- as compared with VEGFsiRNA-treated tumors (Fig. 3*b*). Only a few number of studies describe the use of RNA interference *in vivo* by retroviral (9) or hydrodynamical (10, 11) procedures. We demonstrate here the efficient delivery into tumors of siRNA by systemic i.p. administration at low doses (125 μ g/kg/day) in saline. This treatment resulted in a robust inhibition of the endogenous VEGF expression and produced a marked tumor growth inhibition. No side effects were detected on the living animals or on their organs observed at the end of the experiment.

The efficiency of i.p. administration of VEGFsiRNA on tumor growth inhibition was compared with the effect of TSP1 expression by the tumor cells. A third group of mice (group C) received no dox supply, ensuring the expression of TSP1 and luciferase, and was injected with LUCsiRNA. The growth curves of tumors from groups B and C were very similar (Fig. 3*a*). The VEGF dosage indicated that tumors from group C grew from cells overexpressing VEGF 2.7-fold as compared with tumor cells growing in the absence of TSP1 (Fig. 3*b*). The tumors in the control group reached a volume of 100 mm³ 9.7 \pm 0.9 days after the injection of the cells and 3 days later when TSP1 was expressed (12.7 \pm 0.7 days) or VEGF inhibited (12.7 \pm 0.5 days) in the tumors. The mean of the tumor doubling times in untreated animals (40.4 \pm 2.5 h) was not significantly modified by TSP1 (41.0 \pm 4.8 h) or VEGFsiRNA treatments (44.4 \pm 1.7 h; Fig. 3*a*, *Inset*). These data demonstrate that as previously observed for TSP1 expression, VEGF inhibition by siRNA delays the onset of the tumors but has no effects on the growth rates of the tumors.

To test the efficiency of i.p. administration of VEGFsiRNA on VEGF expression that is triggered by the *in vivo* development of resistance to TSP1 expression, finally, in a fourth group (D), the two treatments were combined: JT8 tumors were grown in animals receiving no dox and injected with VEGFsiRNA. The inhibition of the tumor growth observed in this group was not better than that obtained with VEGFsiRNA alone or TSP1 alone (Fig. 3*a*). We suspected that

TSP1 might be reducing the accessibility of the siRNA to the tumors by limiting tumor vascularization. Luciferase expression was then measured in the tumors from groups C and D. As expected, in the absence of dox treatment, the luciferase activity, which parallels TSP1 expression, was increased 6.8-fold in tumors from group D as compared with group A. Although the animals in group C were injected with LUCsiRNA, no decrease in the luciferase activity was observed in tumors from this group, and only a 23% reduction in VEGF expression was triggered by VEGFsiRNA treatment in group D as compared with group C (Fig. 3*b*). This strongly suggested that the siRNA was not penetrating the tumor properly. This result points out the difficulties likely to be encountered whenever one adds a systemic treatment to an antiangiogenic therapy and offers an explanation for why tumors injected daily with VEGFsiRNA are eventually able to grow.

Synergistic Effects of TSP1 and VEGF siRNA to Inhibit Tumor Growth.

To circumvent this difficulty of delivering systemic siRNA to tumors in the presence of TSP1, JT8 cells were transfected with VEGFsiRNA or LUCsiRNA and injected to nude mice receiving no dox treatment, thus expressing TSP1. On day 16, the mean tumor volume of the control group was 799 ± 270 mm³ (Fig. 4), very close to the values observed in the previous experiment (Fig. 3*a*, group C). This volume was reduced by 86% when the cells were unable to produce VEGF efficiently because of the presence of VEGFsiRNA (mean tumor volume, 111 ± 70 mm³). In these barely palpable tumors, the VEGFsiRNA induced an 82% reduction in VEGF expression (9.8 ± 2.7 versus 56.4 ± 23.9 pg/mg protein). Cells that grew *in vivo* despite these treatments could be untransfected cells, cells re-

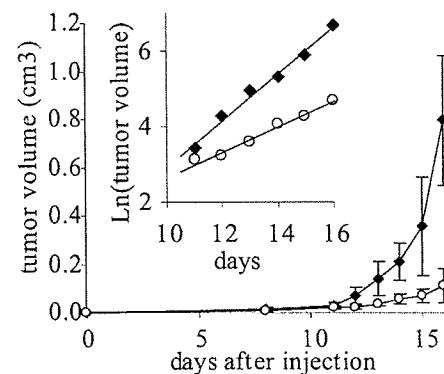


Fig. 4. Synergistic effects of TSP1 and VEGFsiRNA on tumor growth. JT8 cells were transfected with CONTsirNA (◆) or VEGFsirNA (○) and implanted in nude mice receiving no dox supply, thus expressing TSP1. Tumor growth was monitored daily (mean volume \pm SE, $n = 6$). *Inset*: corresponding logarithmic regression trend lines.

expressing VEGF once the siRNA effect is diluted by exponential cell divisions or cells triggering angiogenesis by elaborating other angiogenic factors such as basic fibroblast growth factor. The tumors in this group reached 100 mm^3 15.2 ± 1.2 days after injection of the tumor cells, indicating additive effects of TSP1 and VEGFsRNA to delay the onset of tumors. Remarkably, the logarithmic regression analysis of the growth curves show that VEGFsRNA also reduced the growth rate of the tumors (Fig. 4, *inset*). The doubling time of tumors treated with TSP1 alone (41.9 ± 2.7 h) was increased by 65% when VEGFsRNA were used in combination with TSP1 (69.3 ± 10.3 h). This combination thus not only delays by 6 days the onset of the tumors but also significantly slows down their growth, a parameter that was not affected by the administration of one single treatment.

Discussion

Numerous antiangiogenic agents are currently in clinical trials. Monotherapies in these assays have been disappointing, and several companies have abandoned drugs targeting VEGF or its receptors (13). Resistance, similar to that which has been documented in mice, could be responsible for the failures of these promising agents to consistently curtail human tumor growth. If so, combinational therapies appear to be needed (14, 15). The joining of the antiangiogenic protein endostatin with an antisense strategy against the epidermal growth factor receptor (expressed on a fraction of human tumor cells) has been shown to produce synergistic inhibitory effects on tumor growth (16). In this study, we show that the combination of two different antiangiogenic agents can produce synergistic and not only additive effects to significantly minimize tumor resistance and tumor growth rate. Our finding that it is possible to use siRNA *in vivo* in such studies should facilitate additional efforts to define more efficient sets of treatments. Moreover, siRNA may be used to suppress expression of point mutated genes frequently appearing during the natural history of cancers in humans (17). It is not clear today if the uptake of siRNA in a tumor is facilitated as compared with normal tissues, and biodisponibility studies will have to be performed to address this question. Because of their transient effects *in vivo* and their capacity to penetrate a tumor when injected via a systemic route, it may eventually be possible to use siRNAs sequentially to target one after the other different key proteins in the development of cancer and thereby keep tumors under control.

Acknowledgments

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References

- Rastinejad, F., Polverini, P. J., and Bouck, N. P. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell*, **56**: 345–355, 1989.
- Boehm, T., Folkman, J., Browder, T., and O'Reilly, M. S. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature (Lond.)*, **390**: 404–407, 1997.
- Filleur, S., Volpert, O. V., Degeorges, A., Voland, C., Reicher, F., Clezardin, P., Bouck, N., and Cabon, F. *In vivo* mechanisms by which tumors producing thrombospondin 1 bypass its inhibitory effects. *Genes Dev.*, **15**: 1373–1382, 2001.
- Vilaria-Petit, A., Crombet, T., Jothy, S., Hicklin, D., Bohlen, P., Schlaeppi, J. M., Rak, J., and Kerbel, R. S. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies *in vivo*: a role for altered tumor angiogenesis. *Cancer Res.*, **61**: 5090–5101, 2001.
- Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature (Lond.)*, **411**: 494–498, 2001.
- Shi, Y. Mammalian RNAs for the masses. *Trends Genet.*, **19**: 9–12, 2003.
- McManus, M. T., and Sharp, P. A. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.*, **3**: 737–747, 2002.
- Brummelkamp, T. R., Bernards, R., and Agami, R. A system for stable expression of short interfering RNAs in mammalian cells. *Science (Wash. DC)*, **296**: 550–553, 2002.
- Brummelkamp, T. R., Bernards, R., and Agami, R. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell*, **2**: 243–247, 2002.
- Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A., and Herweijer, H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.*, **32**: 107–108, 2002.
- McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A. RNA interference in adult mice. *Nature (Lond.)*, **418**: 38–39, 2002.
- Mettouchi, A., Cabon, F., Montreau, N., Vernier, P., Mercier, G., Blangy, D., Tricoire, H., Vigier, P., and Binetruy, B. SPARC and thrombospondin genes are repressed by the c-jun oncogene in rat embryo fibroblasts. *EMBO J.*, **13**: 5668–5678, 1994.
- Garber, K. Angiogenesis inhibitors suffer new setback. *Nat. Biotechnol.*, **20**: 1067–1068, 2002.
- Kerbel, R., and Folkman, J. Clinical translation of angiogenesis inhibitors. *Nat. Rev. Cancer*, **2**: 727–739, 2002.
- O'Reilly, M. S. Targeting multiple biological pathways as a strategy to improve the treatment of cancer. *Clin. Cancer Res.*, **8**: 3309–3310, 2002.
- Li, M., Ye, C., Feng, C., Riedel, F., Liu, X., Zeng, Q., and Grandis, J. R. Enhanced antiangiogenic therapy of squamous cell carcinoma by combined endostatin and epidermal growth factor receptor-antisense therapy. *Clin. Cancer Res.*, **8**: 3570–3578, 2002.
- Martinez, L. A., Naguibneva, I., Lehrmann, H., Vervisch, A., Tchenio, T., Lozano, G., and Harel-Bellan, A. Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways. *Proc. Natl. Acad. Sci. USA*, **99**: 14849–14854, 2002.